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Diverse Effects of Stat1 on the Regulation of hsp90α Gene Under Heat Shock

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Abstract Stat1 has been known as a regulator of gene expression and a mediator of IFNγ signaling in mammalian cells, while its effect in a heat shock response remains unclear. We used RNAi knockdown, point mutations, ChIP and promoter activity assays to study the effect of Stat1 on the heat-shock induction of the hsp90α gene under heat shock conditions. We found that Stat1 regulates the heat shock induction of its target genes, the hsp90α gene in a heat shock response while the constitutive activity of the gene remains unaffected. The result of Stat1 in complex with Stat3 and HSF1 that bound at the GAS to lead a moderate heat shock induction was designated as an ''intrinsic'' induction of the hsp90α gene. Additionally a reduced or an elevated level of heat shock induction was also controlled by the Stat1 on hsp90α. These diverse effects on the hsp90α gene were a ''reduced'' induction with over-expressed Stat1 elicited by transfection of wild-type Stat1 or IFNγ treatment, bound at the GAS as homodimer; and an ''enhanced'' heat shock induction with a mutation-mediated prohibition of Stat1/GAS binding. In conclusion, the status and efficacy of Stat1 bound at the GAS of its target gene are pivotal in determining the impact of Stat1 under heat shock. The results provided the first evidence on the tumor suppressor Stat1 that it could play diverse roles on its target genes under heat shock that also shed lights on patients with fever or under thermotherapy. J. Cell. Biochem. 102: 1059–1066, 2007.

Key words: Stat1; heat shock; hsp90α; GAS; gene expression; siRNA; Chromatin IP; IFNγ; Jurkat; U3A

Signal transducers and activators of transcription (Stats) were first known as latent in the cytoplasm and activated in the nucleus. Upon tyrosine phosphorylated by Janus Kinases, Stats were dimerized that entered the nucleus and bound to the target genes as transcription activators [Darnell et al., 1994]. Stat family members may play diverse or even counteract roles in cell growth, apoptosis and differentiation while binding to a DNA consensus of $TT(N)_{5-6}AA$ [Darnell, 1997; Bromberg and Darnell, 2000]. Stat1 is a tumor suppressor that specifically inhibits cell growth, promoting cell cycle arrest and inducing apoptosis [Bromberg and Darnell, 2000]. As a dominant mediator in IFN-γ signaling, Stat1 leads to immune and pro-inflammatory effects in the cells [Chin et al., 1996; Stark et al., 1998; Bromberg and Darnell, 2000]. In this regard, a prerequisite for Stat1 activation of gene transcription is the efficient binding of the Stat1 to the GAS (interferon Gamma Activated Sequence) element $TT(N)_4AA$, in the IFN-γ activating genes [Darnell, 1997; Stark et al., 1998; Ramana et al., 2000]. Consequently, the wild type GAS in the genome is critical for Stat1 binding to direct an appropriate gene transcription in the downstream of IFN-γ signaling pathway [Darnell et al., 1994; Stark et al., 1998; Bromberg and Darnell, 2000; Ramana et al., 2000].

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There are two cytoplasm isoforms of Hsp90 with high homology, the Hsp90α and Hsp90β in the human and other mammalian cells. Despite the close relationship between these proteins, the encoding genes, *hsp90α* and *hsp90β* are distinct and map, as of the humans, to the chromosome 14 and 6 respectively [Hickey et al., 1989; Rebbe et al., 1989; Sreedhar et al., 2004]. Accordingly, the expression and control mechanisms of the two hsp90 genes are diverse both in *cis* and in *trans* [Shen et al., 1997; Zhang et al., 1999; Wu et al., 2003; Zhang et al., 2004]. It was reported earlier [Stephanou et al., 1999] that Stat1 played an enhanced role on the expression of both the hsp70 and the hsp90β genes in fibroblasts treated with γ-interferon (IFNγ). Our preliminary studies showed a diverge effect in that ectopic Stat1 inhibited the expression of a third heat shock gene, the hsp90α in the heat shocked Jurkat cells [Chen et al., 2001]. It was not unusual to have found Stat1 played diverse roles on its target genes since each gene may carry one's exclusive regulatory element(s) and responsiveness to an inducer [Darnell, 1997; Bromberg and Darnell, 2000; Ramana et al., 2000]. Nevertheless, it is still of interest to explore the mechanistic aspects of the Stat1 functions, while being phosphorylated under heat shock, on the induction of the hsp90α gene.

MATERIALS AND METHODS

Cell Culture and Treatments

Human Jurkat cells and SH-SY5Y cells were cultured following the description in the ATCC catalog. U3A cells (from Dr. George R. Stark, Cleveland Clinic Foundation) and U3A-pSG91 cells (from Dr. Xinyuan Fu, Indiana University) were incubated as described elsewhere [McKendry et al., 1991; Chin et al., 1996]. For heat shock, cells were incubated at 42°C for 1 h. IFN-γ (Cytolab Ltd.) treatments were as the follows: Jurkat cells: 50 ng/ml for 12 h; U3A and U3A-pSG91: 10 ng/ml for 5 and 12 h for RNA and protein detection, respectively [Ouchi et al., 2000].

Antibodies

Polyclonal antibodies against p-Tyr^{701}-Stat1 and Stat1 were obtained from Dr. Xinyuan Fu; antibody against HSF1 was from Dr. Carl Wu (NCI, Bethesda); antibody against Stat3 was from Dr. Ke Shuai (University of California, Los Angeles) and antibodies against Stat1 and GAPDH were from Santa Cruz and Chemicon, respectively.

Plasmids

Expression plasmids for Stat1 and Stat1-Y701F were from Dr. Xinyuan Fu and that for PIAS1 was from Dr. Ke Shuai [Liu et al., 1998]. Two reporter constructs of hsp90α-CAT (−1756/+37 and −1462/+37) and a transfection control plasmid (pM-CAT) were constructed as previously described [Zhang et al., 1999; Lu et al., 2005]. Site-directed mutagenesis of the GAS of hsp90α (5′-AAGTTCCTACAAGTG-3′, −1615/−1601) was performed following the instructions in the TransformerTM site-directed mutagenesis kit (Clontech). The TTCC were mutated to CCAG utilizing a mutagenic primer (5′-CTGCGGTAGGAAAAGCCAGTACAAGTGAGGAGAG-3′) and a selective primer (5′-GACTGGTGAGGCCTCAACCAAGTC-3′). The mutation was confirmed by DNA sequencing.

RNA Interference

The siRNA sequence (GGATAATTTTCAGGAAGAC) corresponding to nucleotide positions 623–642 of human STAT1 mRNA (NM_007315) was synthesized by Ambion (Austin, TX). Fluorescein-modified Luciferase GL2 control siRNA was synthesized by Dharmacon (Lafayette, CO). Co-transfection of Jurkat cells with Stat1 siRNA (100 nM) and fluorescein labeled GL2 control siRNA (15 nM) was carried out using Lipofectamine 2000 (Invitrogen). At 2 h after transfection, fluorescence-positive cells were sorted by FACS, and recovered in the growth medium for 48 h.

Quantitative Real-Time PCR Analysis

Total RNAs were extracted from cells and followed by reverse-transcription with first-strand RT-PCR kit (Promega). PCR was performed with Brilliant® SYBR Green QPCR Master Mix (Stratagene) using the DNA Engine Opticon2 Continuous Fluorescence Detection System (MJ Research). PCR were carried out with primers for human hsp90α (5′ primer: 5′-TGGTTGTGTCAAACCGATTG-3′ and 3′ primer: 5′-GTAGTTGTCATGCCATACAG-3′), and human *gapdh* (5′ primer: 5′-GAAGGTGAAGGTCGGAGTC-3′ and 3′ primer: 5′-GAAGATGGTGATGGGATTT-3′). The relative expression level of hsp90α was normalized against *gapdh* using the comparative C_T method recommended by the instrument producer.

Detection of Promoter Activity

Transfections were carried out using electroporation (Gene Pulser II, Bio-Rad). Each reporter plasmid, mixed with pM-CAT at a 9:1 molar ratio, was co-transfected into cells. Cells were recovered 48 h after transfection. The total RNA was extracted and used for detecting the CAT mRNA level in a competitive RT-PCR-based system [Wu et al., 2003; Lu et al., 2005].

Immunoprecipitation and Immunoblot Analysis

Whole cell extracts (WCE) were prepared as described [Xiao and Lang, 2000]. A co-immunoprecipitation assay was carried out on cell lysates (~500 μg of protein) incubated with 2 μl of antibody for 2 h at 4°C. Twenty microliters of Protein A (or G)-agarose (Santa Cruz) was added and incubated at 4°C overnight. Pellets were washed three times with RIPA buffer [Xiao and Lang, 2000], followed by 40 μl of 1× Laemmli buffer suspended and boiled for 5 min. Samples were separated in 8% SDS-polyacrylamide gels and analyzed by Western blotting [Wu et al., 2003].

Chromatin Immunoprecipitation (ChIP) Assay

Jurkat cells were treated, with or without IFN-γ, followed by heat shock at 42°C or were cultured at 37°C for 1 h. ChIP assays were then carried out with formalin cross-linking as previously described [Kuo and Allis, 1999; Zhang et al., 2004]. Primers for amplification of the GAS element of hsp90α gene were 5′-CTGGCAATGGCAGAAACTG-3′ (forward, −1642/−1624) and 5′-GAATCCGGAAGCAGGAAGAG-3′ (reverse, −1485/−1504). Primers for control HSEC were 5′-GCGGCGATTGAGGGAAGGT-3′ (forward, −223/−205) and 5′-GGGACGCTGAAGCAACTGACG-3′ (reverse, −3/+18). The lengths of the PCR products were 158 and 241 bp, respectively. For detection of the immunoprecipitated proteins from the chromatin, the protein fractions recovered from the protein–DNA complex were eluted from Protein-A (or -G)-beads, followed by standard procedures for Western blot assay.

RESULTS AND DISCUSSION

The Regulatory Role of Stat1 on hsp90α Gene Under Heat Shock

As Stat1 requires translational modifications for activation [Darnell, 1997], we first examined the phosphorylation on tyrosine 701 of Stat1 under heat shock for up to 2 h (Fig. 1A). It was found that the phosphorylation of Stat1 started in the first 15–30 minutes of heat shock suggesting that the dimer formation via phosphorylated tyrosine and the SH2 domain interaction could be induced under heat shock conditions [Darnell, 1997].

To identify the effects of Stat1 and its responding element on the hsp90α gene upon heat shock, promoter activities were determined. We have found that only the −1756/+37 but not the −1462/+37 fragment of hsp90α gene was affected by either ectopic PIAS1 [Liu et al., 1998] or the $Stat1^{Y701F}$ mutant (Fig. 1B, left panel). Meanwhile, the effect of the $Stat1^{Y701F}$ mutant was more efficient than the ectopic PIAS1 (filled bars, groups 2 and 3) on enhancing the heat shock induction of the gene. The variation could be a result of the multiple functions of PIAS1 beyond being simply an inhibitor of Stat1 [Shuai, 2000; Rogers et al., 2003; Liu et al., 2005], as compared with the highly specific point mutation construct of Stat1. The results suggest that there is a critical heat shock responding element to Stat1 within the −1756/−1463 region of the hsp90α gene.

GAS is the Critical Element of hsp90α Gene That Responds to Stat1 Under Heat Shock

Analyzing the DNA sequences between −1756/−1463, a GAS element TTCCTACAA was found at −1612/−1605 in the hsp90α gene (Fig. 1C, top row). Point mutations of the TTCC to CCAG within the GAS element were performed and are represented as "GXS" to determine the specific requirement of the GAS on the Stat1 regulation on hsp90α upon heat shock (Fig. 1C, bottom row).

We found that Stat1 could conduct three 'level's of heat shock induction on the hsp90α gene: (1) A moderate, 2–3-fold heat shock induction of hsp90α gene with endogenous Stat1 (Fig. 1D filled *vs*. open bars in group 1), designated as an "*intrinsic*" induction of the gene. (2) An obviously reduced heat shock induction of hsp90α gene with wild type Stat1 over-expressed in the cells represented as a "*reduced*" induction of the gene under heat shock conditions (Fig. 1D filled *vs.* open bars in group 2). (3) An efficient heat shock-induction of the hsp90α gene over 4–5-fold with the ectopic mutant of $Stat1^{Y701F}$ designated as an "*enhanced*" heat shock induction (Fig. 1D filled

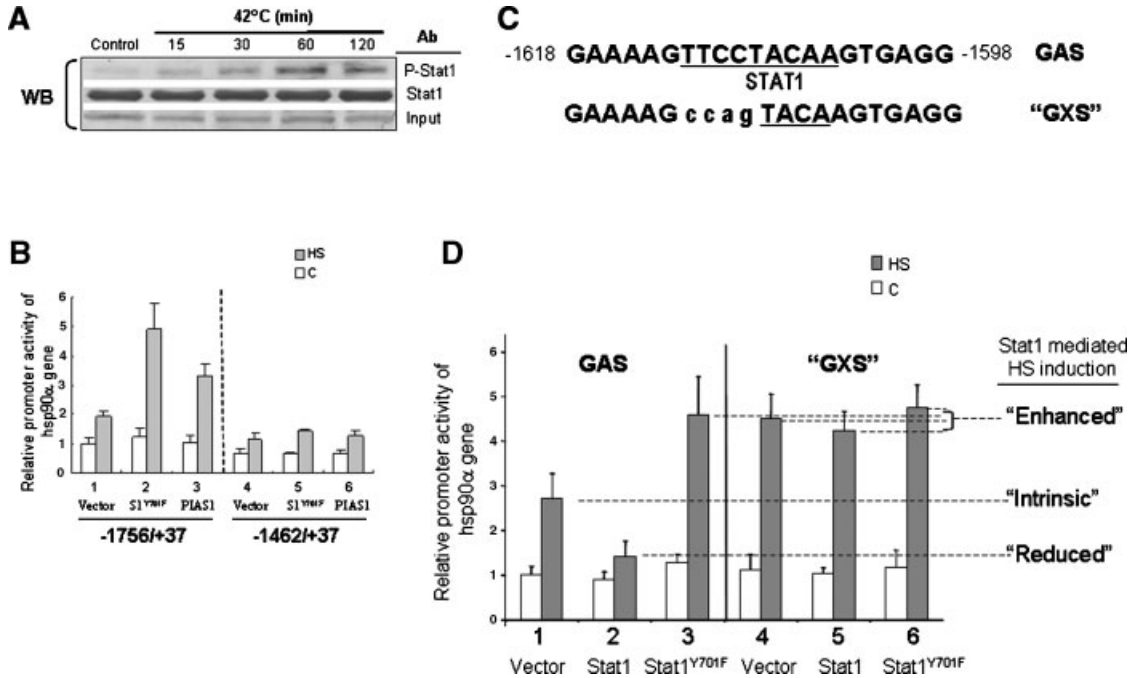

Fig. 1. Effects of Stat1 and GAS on the expression of hsp90α gene under heat shock. **A**: Heat shock induced tyrosine phosphorylation of Stat1 in Jurkat cells. Control:37°C, Heat shock: incubated at 42°C for the time specified. WB: Western Blot. Ab:blotted antibodies for phosphorylated tyrosine of Stat1 (P-Stat1) and pan Stat1 (Stat1). Input: protein loading control. **B**: Promoter activity assay of hsp90α gene in Jurkat cells. Control vector, Stat1^{Y701F} and PIAS1 were individually transfected as indicated. Open bars: 37°C; filled bars: 42°C. Each bar is shown as the mean ± S.D. from at least three independent experiments. **C**: Sequences of the GAS element of the hsp90α gene. Stat1 binding sequences at −1612/−1605 (underlined). ''GXS'':point mutation sequences of ccag in the GAS. **D**: Promoter activity assay of the wild type GAS and mutant ''GXS'' of the hsp90α gene. CAT Reporter construct was driven by the −1756/+37 fragment of the gene. Control vector, Stat1 and Stat1^{Y701F} were individually transfected as indicated. Details were the same as described under part B. Levels of heat shock induction are indicated by broken lines with designations labeled on the right.

vs open bars in group 3). However, the "GXS" mutated promoter of the hsp90α gene (Fig. 1D, filled bars, groups 4–6) showed an enhanced heat shock induction that were comparable to the level of the ectopic Stat1^{Y701F} with the GAS promoter (filled bar of group 3). These data indicate that the heat shock induced phosphorylation and dimerization of Stat1 and its binding to the GAS are critical in heat shock induced promoter activity of the hsp90α gene. This effect could possibly be a part of the Stat1 function as a tumor suppressor against the growth progression usually seen in the presence of Hsp90 proteins [Bromberg and Darnell, 2000; Broemer et al., 2004; Sreedhar et al., 2004; Zhang et al., 2004].

Reduction Mechanism of the hsp90α Gene Under Heat Shock Via IFNγ Treatment or Ectopic Stat1

As Stat1 can be induced by IFNγ in Jurkat and other cell types (supplementary figures) and reported elsewhere [Darnell et al., 1994; Ramana et al., 2002], it was important to explore whether the inhibitory role of Stat1 on the heat shock induction of hsp90α gene might also be a downstream effect of IFNγ. We detected the "intrinsic" mRNA expression of the hsp90α gene upon heat shock that was some six-folds higher than its counterpart at normal temperature (Fig. 2A, group 1, filled *vs* open bars); by contrast, IFNγ treatment completely abolished the heat shock induction of the gene in Jurkat cells (Fig. 2A, filled bars, groups 2 *vs* 1). To our surprise, the heat shock induction of the gene in Stat1 knockdown cells was enhanced either with or without IFNγ treatment (Fig. 2A, filled bars, groups 4 and 3 vs. groups 2 and 1). The results support the contention that the heat shock induction of hsp90α gene is an event in the downstream of IFNγ signaling and is regulated via Stat1.

As both of the IFNγ treatment (Fig. 2A, group 2) and the wild-type Stat1 over-expression (Fig. 1D, group 2) reduced the heat shock induction of the hsp90α gene in Jurkat cells, it

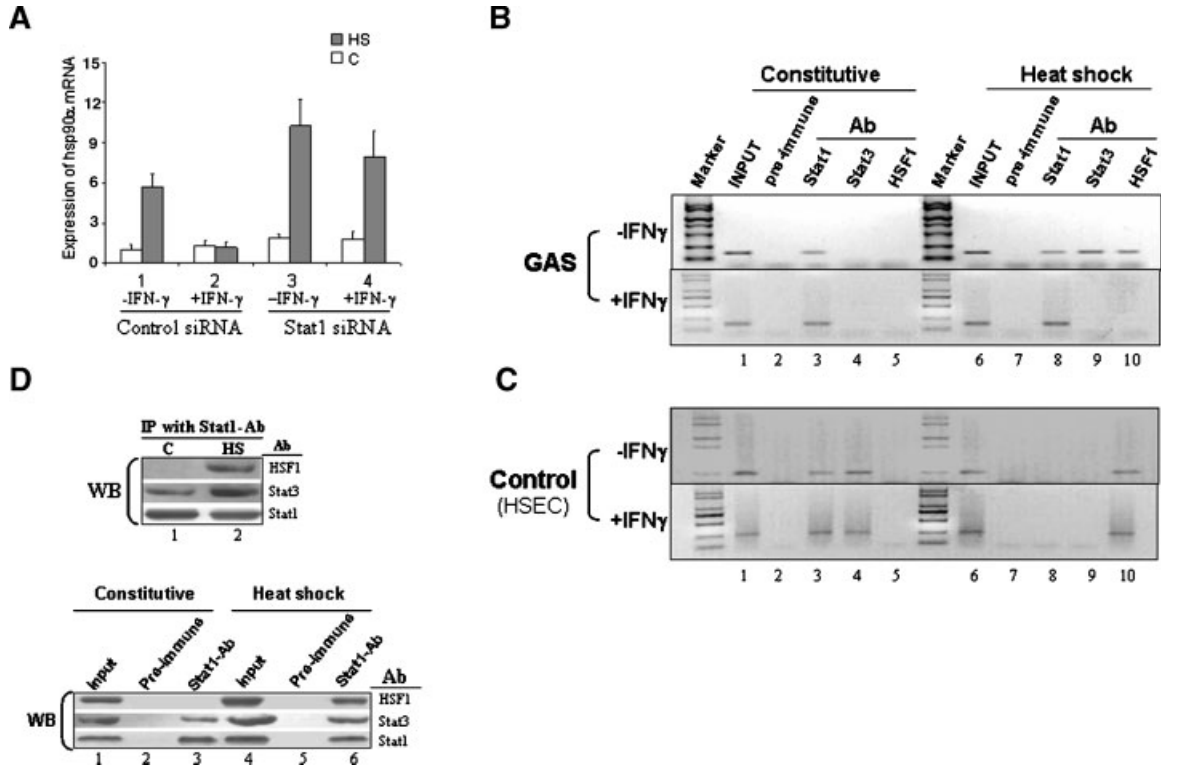

Fig. 2. The impact of IFN-γ or Stat1 on the regulation of hsp90α gene under heat shock. **A**: Real-time RT-PCR assay of hsp90α mRNA in Jurkat cells. Cells transfected with control siRNA (left half) and Stat1 siRNA (right half). ''+'' or ''−'' IFN-γ: with or without IFN-γ treatment. Other descriptions are the same as under (Figure 1B). **B**: ChIP assays of the GAS element in hsp90α gene. ''+'' or ''−'' IFN-γ: with or without IFN-γ treatment. Serum: pre-immune antibody, Input: chromatin without antibody precipitation. Ab: antibodies used in ChIP assay. **C**: ChIP assay control (HSEC): a non Stat-mediated heat shock responsive element at −223/+18 of the hsp90α gene. Descriptions are the same as under part B. **D**: Immunoprecipitation assay for proteins from whole cell extract (upper panel) and chromatin (lower panel) precipitated with Stat1. C: 37°C; HS: 42°C. The immunoprecipitated proteins were separated on SDS-PAGE and blotted with antibodies as indicated subsequently after each stripping. Pre-immune: serum used as negative controls (lower panel, lanes 2 and 5); chromatin samples reverse cross-linked without adding antibody are designated as Input (lanes 1 and 4).

was of interest to explore whether the underlying mechanisms were similar. In ChIP assays, we found that Stat1 was consistently bound to the GAS element in IFNγ treated cells under heat shock (Fig. 2B, lanes 3 and 8, bottom row), which was likely to be responsible for the abolishment of the heat shock induction of the hsp90α gene (Fig. 2A, filled bars, group 2 vs. group 1). As Stat1 consistently bound at GAS under IFNγ treatment and that Stat1 knockdown could counteract the IFNγ repression in heat-shocked cells, which suggested that the repression effect of IFNγ on the hsp90α gene was mediated by the homodimer Stat1 bound at GAS of the gene.

It was found that endogenous Stat1 alone bound to the GAS without heat shock, by contrast, with heat shock it was in complex with Stat3 and HSF1 at the GAS site (Fig. 2B, upper row, lanes 3 vs. 8–10). Co-immunoprecipitation assays showed that Stat1 was in complex with Stat3 and HSF1, in both the whole cell extract (Fig. 2D, top panel) and chromatin complexes (bottom panel). The Stat1 and Stat3 interaction found in the non-heat shock control of Figure 2D (top panel, lane 1; bottom panel, lane 3) was unrelated to the heat shock-induction of hsp90α gene, as it only bound to a proximal HSE complex at −96/−60 in the constitutive expression of the hsp90α gene (Fig. 2C, *Control* (HSEC) panel, lanes 3 and 4). In addition, HSF1 may be recruited by Stat1 and/or Stat3 to the GAS of the hsp90α gene, as there is only one 'gTTCc' in the GAS element that matches the typical 5 nucleotide motif (nGAAn) of the known functional heat shock element. In other words, HSF1/GAS binding could be 1/1,000 times less efficient than that of a typical HSF/HSE binding, which consists of successive motifs as seen in the

nGAAnnTTCnnGAAn sequences [Sorger, 1991; Lis and Wu, 1993].

These data suggest that: (1) the endogenous Stat1 induced by heat shock interacted with Stat3 to form a heterodimer at the GAS element and recruited HSF1 to elicit an "intrinsic" activation of hsp90α gene under heat shock; (2) IFNγ induced elevation of Stat1, similar to the ectopic Stat1, solely exists as homodimer at the GAS site (Fig. 2B, bottom panel) that neither interacts with Stat3 nor recruits HSF1 upon heat shock and leads to a reduced heat shock-induction of the hsp90α gene. The results confirmed that IFNγ played a similar role with the over-expressed wild type Stat1 on the reduced expression of the hsp90α gene under heat-shock.

The Diverse Effects of Stat1 on the Heat Shock Induction of hsp90α Gene in Other Human Cell Types

We also examined the effect of Stat1 on the hsp90α gene under heat shock in a Stat1 null cell (U3A) and its Stat1 re-established counterpart, U3A-pSG91 cells. It was found that the mRNA expression profiles of hsp90α gene in heat shocked U3A cells with IFNγ treatment was exactly the same as that of Stat1 knock-down Jurkat cells under heat shock (Fig. 3A, filled bars, groups 1 and 2 and Fig. 2A, filled bars, groups 3 and 4). Further, the effects of IFNγ shown in the Stat1 re-established U3A cells were comparable with those found in wild type Jurkat cells (filled bars in Fig. 3A, groups 3 and 4 vs. Fig 2A, groups 1 and 2).

A neuroblastoma cell line SH-SY5Y that constitutively expresses Stat1, showed similar diverse levels to those found in Jurkat cells (Fig. 1D) in terms of "intrinsic", "reduced" and "enhanced" effects under heat shock (filled bars, Fig. 3C). By contrast, as a Stat1-null cell type, the heat-shocked U3A cells showed no "intrinsic" level of hsp90α gene expression but rather an "enhanced" level, in the case of vector transfection (Fig. 3B, filled bar of group 1). These results suggest that Stat1 is an indispensable general factor that appropriately controls the expression of its target gene, not only under physiological conditions, but also as a result of heat shock.

Summarizing the above, the existence of both an activated Stat1 and a non-mutated GAS is the basic requirements for an appropriate expression of hsp90α gene under heat shock. Therefore, genetic mutations, if any, either in Stat1 or the GAS could produce serious outcomes in vivo via an inadequate expression of the hsp90α gene. In addition, the stringent control on the expression of the hsp90α is of significance, since it has been found that Hsp90α could be secreted out of the cell where it could activate the matrix metalloproteinase 2 that eventually enhances cancer invasion or metastasis [Eustace et al., 2004]. The novel Stat1/GAS pathway for the control of the heat shock induction of the hsp90α gene shown here suggests that IFNγ treatment or ectopic Stat1 could function as a novel means of blocking cancer metastasis.

In conclusion, we have shown for the first time that Stat1 is phosphorylated under heat shock and participates as a functional regulator, on at least one of its target genes. The efficiency of the Stat1 induction of the hsp90α gene upon heat

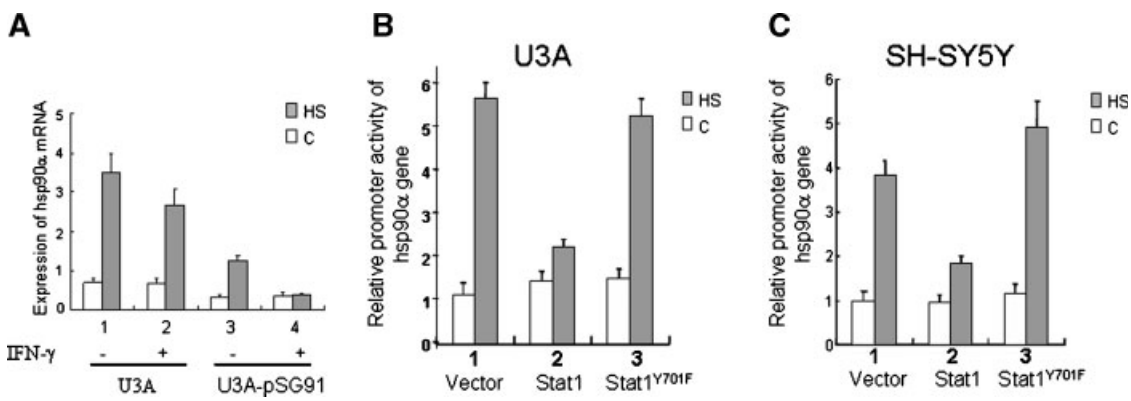

Fig. 3. Effect of Stat1 on hsp90α gene in other human cell lines. **A**: Heat shock induction of hsp90α mRNA in U3A (left) and U3A-pSG91 cells (right). Descriptions are the same as under Fig. 2A. Promoter activity assay of the hsp90α gene in U3A cells (**B**) and in SH-SY5Y cells. (**C**) with or without heat shock. All the treatments and descriptions are the same as for Fig. 1D, groups 1–3.

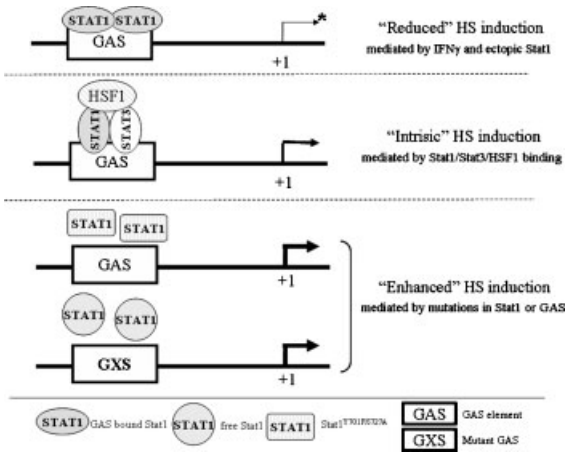

Fig. 4. A schematic model for Stat1 mediates heat shock induced differential expression of *hsp90α* gene. Arrow represents the transcription efficiency of the gene from +1. * also represents the constitutive expression level of hsp90α gene mediated by Stat1 without heat shock.

shock is diverse and depends upon the existence of Stat1, the component(s) in the Stat1 complex or the forms of Stat1 dimer available at the GAS element of the gene, which was summarized and illustrated in Figure 4. The results provided the first evidence on the tumor suppressor Stat1 that it could play diverse roles on its target genes under heat shock that also shed lights on the role of Stat1 in local inflammation and in patients with fever or under thermotherapy.

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